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ORIGINAL ARTICLE

Bacterial invasion into radicular dentine—an in vitro study

Simone Stauffacher[1] · Adrian Lussi[1] · Sandor Nietzsche[2] · Klaus W. Neuhaus[1] · Sigrun Eick[3]

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Abstract
Objectives We wanted to investigate differences in invasiveness into radicular dentinal tubules by monocultured and co-cultured bacteria frequently found in infected root canals.
Methods Fifty-one human roots were incubated for 8 weeks with monocultured *Streptococcus gordonii* ATCC 10558, *Streptococcus sanguinis* ATCC 10556, and with five capnophiles/anaerobes as well as with capnophiles/anaerobes co-cultured with a streptococcal species. Thereafter, bacterial samples were cultured from the inner, middle, and outer third of the root dentine of longitudinally broken teeth ($n = 5$). In addition, scanning electron microscopy (SEM) images were obtained.
Results Single gram-positive species were able to penetrate into the middle and outer third of the root dentine. *Fusobacterium nucleatum* ATCC 25586 was not found in any of the dentine specimens. *Prevotella intermedia* ATCC 25611 and *Porphyromonas gingivalis* ATCC 33277 were found in the inner and middle third.

The bacterial load of streptococci was higher in all thirds in co-cultures compared to single infections. In co-cultures with streptococci, *Actinomyces oris* ATCC 43146 was found in the outer third in 9/10 samples, whereas *P. intermedia* ATCC 25611 was not detectable inside dentine. Co-culture with *S. sanguinis* ATCC 10556 enabled *F. nucleatum* ATCC 25586 to invade dentine; SEM images showed that *F. nucleatum* ATCC 25586 had a swollen shape.
Conclusions Invasiveness of bacteria into dentinal tubules is species-specific and may change depending on culturing as a single species or co-culturing with other bacteria.
Clinical relevance Oral streptococci may promote or inhibit invasion of capnophiles/anaerobes into radicular dentine.

Keywords Bacterial invasion · Root dentine · Scanning electron microscopy · Co-culture · Monoculture · Dentinal tubules

Introduction

Root canal infections are composed of multiple bacterial species; they are dominated by anaerobic gram-positive bacteria and differ significantly between subjects [1–4]. Bacteria are particularly important for the initiation, progression, and persistence of apical periodontitis [5]. A persistent intra-radicular presence of bacteria after chemo-mechanical treatment is considered to be a possible cause of endodontic failure [6]. Matsuo et al. [7] showed that bacteria localized deep inside the dentinal tubules could not be removed by endodontic treatment because they are often protected from the action of endodontic instruments and irrigation solutions. Approximately 100 different bacterial species were identified in post-instrumented and/or post-medicated samples, such as *Streptococcus sanguinis*, *Streptococcus gordonii*, *Fusobacterium nucleatum*, *Parvimonas micra*, *Prevotella intermedia*, *Porphyromonas gingivalis*, and *Actinomyces* sp. [4]. However, sclerotic or obliterated dentinal tubules can hamper bacterial invasion and promote a high rate of

Klaus W. Neuhaus and Sigrun Eick ex aequo contribution

✉ Klaus W. Neuhaus
klaus.neuhaus@zmk.unibe.ch

[1] Department of Preventive, Restorative and Pediatric Dentistry, University of Bern, Freiburgstrasse 7, CH-3010 Bern, Switzerland

[2] Center of Electron Microscopy, University Hospital of Jena, Jena, Germany

[3] Laboratory of Oral Microbiology, Department of Periodontology, University of Bern, Bern, Switzerland

endodontic treatment success [8–10]. Bacteria are able to penetrate into dentinal tubules by cell division [11, 12]. The extent and depth depend on bacterial species-specific characteristics (e.g., bacterial size, invasiveness) and on the tubule diameter [7, 11–14].

Previous data demonstrated that *S. gordonii* and *S. mutans* express antigen I/II polypeptide surface adhesins, which facilitate streptococcal binding to collagen [15]. The presence of collagen type I within dentinal tubules appears to be essential by oral bacteria, because it serves as a substrate for adhesion, stimulates intra-tubular growth, up-regulates production of antigen I/II polypeptide, and induces a morphological growth response (long-chaining of streptococci was observed) [15]. All of these effects facilitate bacterial penetration into dentinal tubules [15]. No penetration into dentinal tubules was observed in isogenetic mutants of *S. gordonii*, which are deficient in the production of the antigen I/II polypeptides SspA and SspB [15]. Early adherent bacteria of the oral cavity (e.g., *S. gordonii*, *S. sanguinis*) coaggregate with other oral bacteria [16]. Binding of *F. nucleatum* to *S. sanguinis* influences surface properties and virulence of *F. nucleatum* [17]. The expression of *S. gordonii* antigen I/II polypeptide (SspA and SspB) mediates the specific adherent interaction between *S. gordonii* and *P. gingivalis*, which enhances the dentinal invasion of *P. gingivalis* [18]. In contrast, antigen I/II polypeptide expressed by *S. mutans* does not have the same binding capacity or invasive impact on *P. gingivalis*, leading to the conclusion that the function of antigen I/II polypeptides is species-specific [18].

The purpose of the present study was to investigate the in vitro invasion into dentinal tubules of monocultured and co-cultured obligate and facultative anaerobic bacteria commonly isolated from root canal infections together with oral streptococci (*S. gordonii*, *S. sanguinis*) over an eight-week incubation period. Furthermore, we wanted to detect gene expression that was associated with invasion into dentinal tubules.

Materials and methods

Specimen preparation

Teeth extracted by dental practitioners over the past year in the Canton of Bern, Switzerland were stored in 1 % chloramine solution. From this irreversibly anonymized pool of human teeth, 51 maxillary molars without deeper coronal or root caries lesions or endodontic treatment were selected for this study. Patients consented to the use of their teeth for research purposes. This procedure complies with the relevant regulations in Switzerland and the local ethics committee.

Initially, the teeth were thoroughly rinsed with water and then cleaned with a toothbrush and fluoride-free pumice. Organic debris was removed using a 2-min application of 3 % NaOCl. In order to obtain single roots with a round-shaped root canal, only the palatal roots were included in this study. After decoronation, the root canals were instrumented 1 mm short of the apical foramen using ProTaper Next instruments (PTN; Dentsply Maillefer, Ballaigues, Switzerland) up to #40.06. The irrigation solution used during instrumentation was 3 % NaOCl, and a final passive ultrasonically activated irrigation with 17 % EDTA was performed for 60 s to remove smear layer. After root canal preparation, the apical foramen was sealed with a self-adhesive flowable composite (Vertise Flow, Kerr, Orange, USA) to prevent bacterial leakage. To minimize an influence by disinfecting solutions, the teeth were stored in 0.9 % *w/v* NaCl solution for 2 months with weekly exchange of the NaCl solution before using in experiments.

The roots were then vertically embedded into silicone blocks (Putty regular set, Provil novo Dose Refill, Heraeus Kulzer GmbH, Hanau, Germany) and autoclaved in a box with distilled water for 20 min at 121 °C.

Inoculation with bacterial species

Seven monocultured microbial strains (three gram-negatives anaerobic rods, one anaerobic gram-positive coccus (*P. micra*), one capnophilic gram-positive rod (*Actinomyces oris*), and two streptococcal strains) were used. In addition to the monocultures, a combination of the five first mentioned species with one out of two *Streptococcus* sp. was prepared (Table 1). All strains were cultured under anaerobic conditions on tryptic soy agar (TSA) plates with 5 % sheep blood. The suspensions of *S. sanguinis* ATCC 10556 and *S. gordonii* ATCC 10558 cells were prepared in 0.9 % NaCl (McFarland 4), afterwards both suspensions were diluted 1:9 with brain heart infusion broth (BHI, Oxoid, Basingstoke, UK) supplemented with 5 mg/L NAD (Sigma-Aldrich, Buchs, Switzerland). The suspensions of *P. intermedia* ATCC 25611, *P. gingivalis* ATCC 33277, *F. nucleatum* ATCC 25586, *A. oris* MG1 (ATCC 43146), and *P. micra* ATCC 33270 cells were prepared directly in Wilkins-Chalgren broth (Oxoid) with NAD. To avoid initial overgrowth by the

Table 1 Tested microbial strains and mixtures

Streptococcus	Other strains
Streptococcus sanguinis ATCC 10556	*Prevotella intermedia* ATCC 25611
Streptococcus gordonii ATCC 10558	*Porphyromonas gingivalis* ATCC 33277
	Fusobacterium nucleatum ATCC 25586
	Actinomyces oris MG1 (ATCC 43146)
	Parvimonas micra ATCC 33270

streptococci, streptococci were mixed with the other strains at a ratio of 1:9.

The boxes containing the teeth were opened in a laminar air flow cabinet. Each tooth was inoculated with 15 μL of suspension corresponding to the 17 groups. The boxes were then placed inside an anaerobic chamber and incubated at 37 °C for 8 weeks. Once a week, new bacteria were added and the bacterial growth as well as the purity were monitored. Nutrient medium (Wilkins-Chalgren broth and NAD) was exchanged every 3 days.

Sampling procedure and bacterial load assessment

After 8 weeks of incubation, the roots were broken longitudinally. The area of the root dentine at the line of breakage was divided horizontally into three parts: the inner third (close to the root canal), the middle third, and the outer third (close to cementum). Out of each third in the longitudinal middle of the root, each sample of dentine chips was collected with a new sterile rose bur (no. 8). Sample collection started from the outer third to avoid any cross-contamination. Thereafter, the bur with debris was placed into an Eppendorf tube filled with 100 μL of Wilkins-Chalgren broth with NAD. Those tubes were vortexed for 10 s before 900 μL of Wilkins-Chalgren broth with NAD was added. Then, the samples were spread on TSA plates with a 10 μL inoculating loop. After 24 h of anaerobic incubation, again 10 μL of Wilkins-Chalgren broth was transferred to TSA plates with 5 % blood agar. After 3–7 days of anaerobic incubation, the growth on agar plates was semi-quantified for each bacterial strain using the following scoring system: no growth; low load, ≤5 colonies or growth only after enrichment in broth; moderate load, ≤50 colonies; high load, >50 colonies. In mixed cultures, strains were identified by colony morphology or by nucleic-acid based methods.

Scanning electron microscopy images

SEM images of one sample per group were obtained to visualize invasion into dentinal tubules. For SEM, the halves of the teeth were broken again and the samples were immediately fixed in 2 % glutaraldehyde in cacodylate buffer for 30 min, washed twice with cacodylate buffer, and dehydrated using a graded ethanol series (10 min each concentration). Following critical point drying, the samples were sputtercoated with gold and examined with an SEM (ZEISS LEO-1530 Gemini, Carl Zeiss, Jena, Germany) equipped with a field emission electron gun at 10 keV.

mRNA expression of A. oris gene fim-P1

To detect differences in gene expression between monocultured and co-cultured strains, primers for *fimP-1* (accession: AF 106034.3) of *A. oris* were designed. *Sod* was used as a housekeeping gene (accession: X81381.1).

After checking primers for *fim-P1* (5′-AGT GCG AAC AGA CCG ACA AT-3′, 5′-TCG GAG ACC TGC TTG TCA AC-3′) and sod (5′-CCT GTG GGA GAA GAA CCT CG-3′, 5′-TCG AGG TAG AAT GCG TGC TC-3′) on DNA, the following experimental design was chosen: *A. oris* was incubated as a single species or mixed with *S. gordonii* and *S. sanguinis* (all overnight cultures OD600nm=1) in cultivation media for 1 and 6 h. Because of the quantity of RNA needed, we were unable to inoculate root canals. Instead, parts of six halves of roots were added to 1 mL of culture. Total RNA was purified using an innuPREP RNA mini kit (Analytic Jena AG, Jena, Germany) and cDNA was synthesized from 100 ng total RNA using an RevertAid RT kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's instructions. Real-time polymerase chain reaction was carried out using GoTaq qPCR Master Mix (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions.

Statistics

The Fisher exact test was used to compare categorical data of single and mixed contaminations and of different groups of bacteria. The Student *t* test was used to compare mRNA expression. The statistical software program used was SPSS (version 22.0, Stanford University, Stanford, California, USA). The level of significance was $\alpha = 0.05$.

Results

A weekly check of the cultures confirmed that there was not any contamination by microorganisms other than the applied ones. Samples taken from the root canal were positive in all cases after at least 6 weeks of incubation.

Invasion of monocultured strains

All tested monocultured bacterial strains except *F. nucleatum* could be detected in at least the inner third of root dentine. *S. sanguinis* ATCC 10556, *A. oris* MG1, and *P. micra* ATCC 33270 were found in the outer third of root dentine. Differences between streptococci, other gram-positive species and gram-negative species were significant for the outer third of the root dentine ($p = 0.047$). In the outer part of the root dentine, *A. oris* MG1 was the only species found at a high load (Fig. 1).

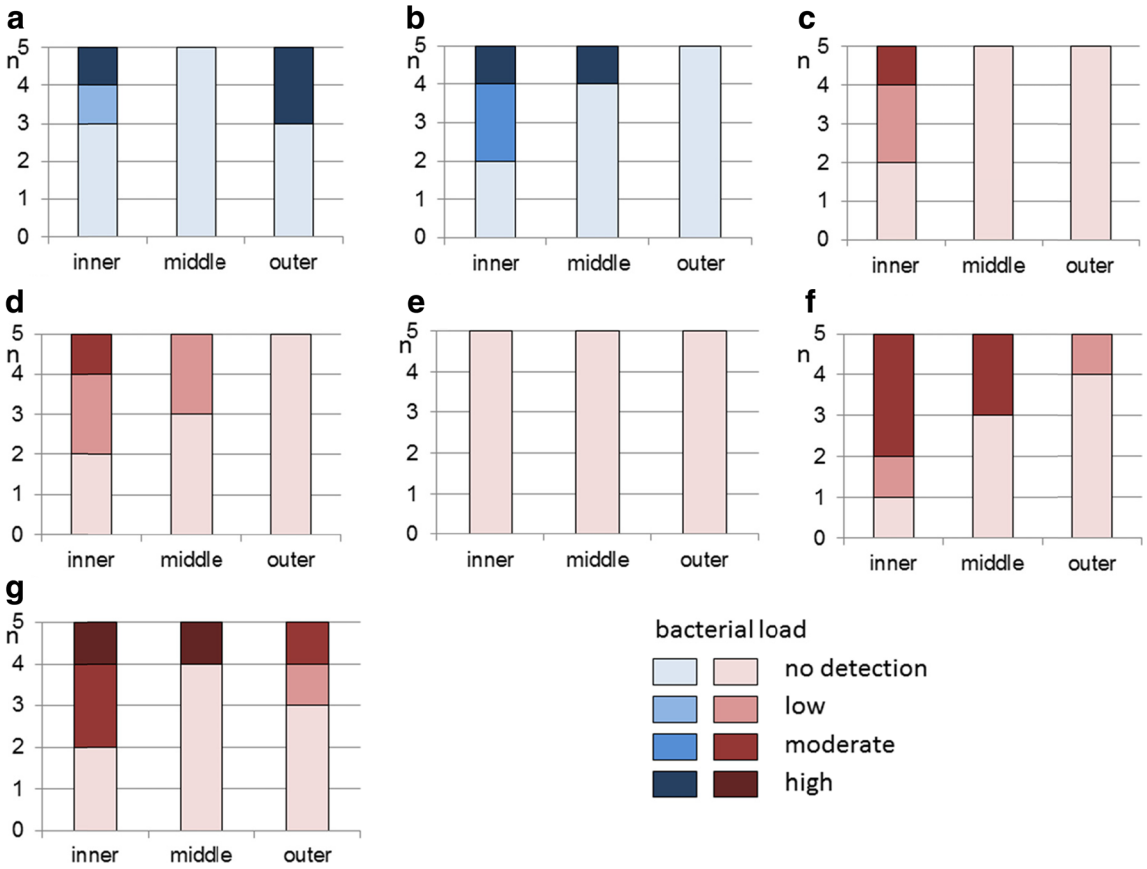

Fig. 1 Categorial data of *Streptococcus sanguinis* ATCC 10556 (**a**), *Streptococcus gordonii* ATCC 10558 (**b**), *Prevotella intermedia* ATCC 25611 (**c**), *Porphyromonas gingivalis* ATCC 33277 (**d**), *Fusobacterium nucleatum* ATCC 25586 (**e**), *Actinomyces oris* ATCC MG1 (**f**), *Parvimonas micra* ATCC 33220 (**g**) in the inner, middle, and outer part of root dentine after 8 weeks of co-cultivation

Invasion of mixed cultured strains

P. intermedia ATCC 25611, *P. gingivalis* ATCC 33277, and *P. micra* ATCC 33270 co-cultured with *S. sanguinis* ATCC 10556 did not penetrate into root dentine. *F. nucleatum* ATCC 25586 was found in two of the five analyzed samples. *A. oris* MG1 was found in the dentinal tubules in the inner and middle third (5/5 each) and in 4/5 halves of root dentine. Most of the samples showed a moderate to high load of *A. oris* MG1 (Fig. 2).

P. intermedia ATCC 25611 and *F. nucleatum* ATCC 25586 were not detected by culture in root dentine when they were co-cultured with *S. gordonii* ATCC 10558. However, in combination with *S. gordonii* ATCC 10558, *A. oris* MG1 invaded the root dentine from the inner to the outer part in 5/5 root halves. Only two of the 15 samples in the outer part of the root dentine yielded low counts of that species (Fig. 3).

There was no significant difference between the two chosen streptococcal strains when tested in combination. Invasion by the streptococci depended on the combined strain (gram-positive vs. gram-negative) in the inner third of the root ($p = 0.049$). Invasion by the combined strain (gram-positive vs. gram-negative) was different in all three thirds (each $p < 0.01$).

Comparison of single and mixed contaminations

Using categorical data, significant differences for invasion between single and mixed infections were found for streptococci in the inner ($p = 0.028$), the middle and the outer thirds (each $p < 0.01$), and for the combined strains in the inner third ($p = 0.014$).

Scanning electron microscopy

Most SEM images confirmed the findings obtained from the cultures. Bacteria were located in high quantities at the entrances and within dentinal tubules as shown for *A. oris* MG1 as a single strain (Fig. 4a, b). The SEM pictures demonstrated that the smear layer could be largely removed through the endodontic therapy so that the dentinal tubules were open for bacterial invasion.

Figure 5a, b shows a mixed contamination of *S. sanguinis* with *F. nucleatum*: around the tubules *F. nucleatum* is visible, whereas in the dentinal tubules mostly cocci (*S. sanguinis*) are found. *A. oris* MG1 in combination with *S. sanguinis* were found to penetrate the dentinal tubules (Fig. 5c, d).

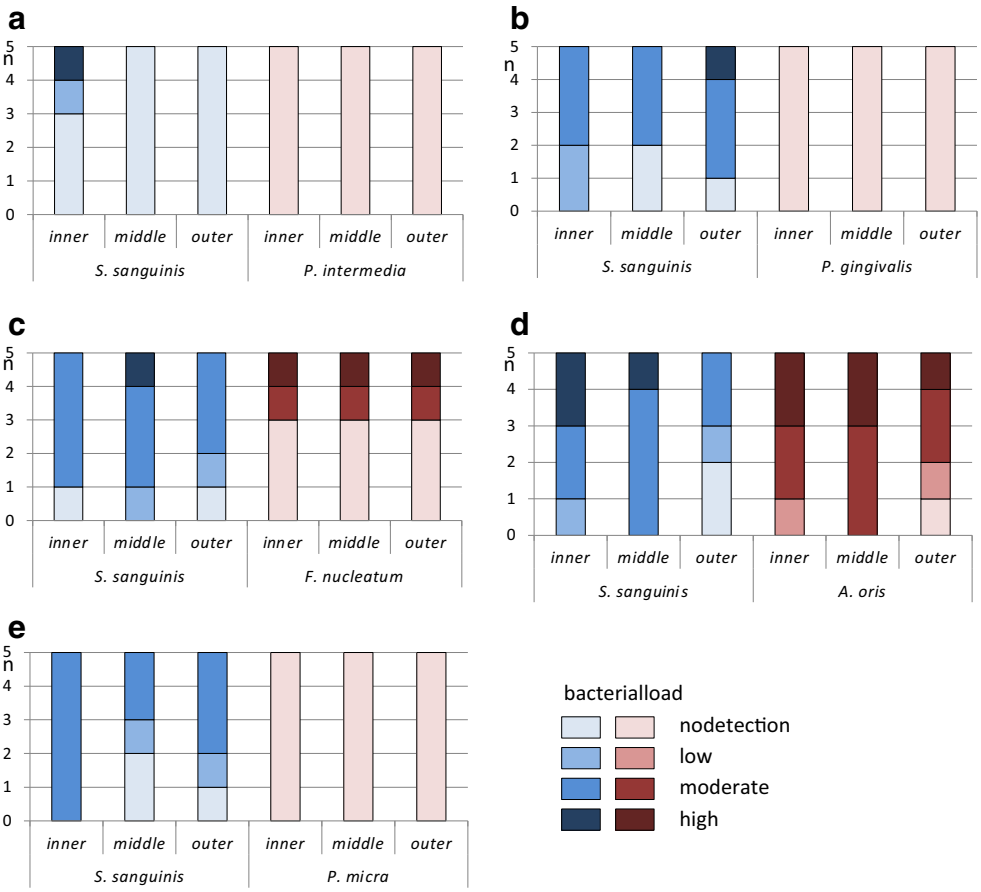

Fig. 2 Categorial data of *Streptococcus sanguinis* ATCC 10556 combined with *Prevotella intermedia* ATCC 25611 (**a**), *Porphyromonas gingivalis* ATCC 33277 (**b**), *Fusobacterium nucleatum* ATCC 25586 (**c**), *Actinomyces oris* MG+ (**d**), *Parvimonas micra* ATCC 33220 (**e**) in the inner, middle, and outer part of root dentine after 8 weeks of co-cultivation

In Fig. 6, strains together with *S. gordonii* are presented. Although not detected by culture in the outer third, *F. nucleatum* is visible in high quantities at the entrances of the dentinal tubules, however its shape is modified (Fig. 6a). Joint contamination with *P. micra* ATCC 33270 shows cocci within the tubules (Fig. 6b). *A. oris* MG1 is clearly detectable within the root dentine (Fig. 6c, d).

mRNA expression of A. oris fim-P1

Expression of *fim*-P1 was related to the 1-h incubation of single *A. oris* without root halves. At 1 h, co-incubation resulted in increased expression (not significant) in combination with *S. gordonii* but resulted in no change in combination with *S. sanguinis*. After 6 h, expression dropped for the mixed culture incubated without teeth (with *S. gordonii*: $p = 0.001$, with *S. sanguinis*: $p = 0.002$).

At 1 h, a decrease in expression to approximately 33 % ($p = 0.035$) was observed when root halves were added to a single strain. However, the root halves did not appear to affect expression when *A. oris* was combined with *S. gordonii* or *S. sanguinis*. At 6 h, in co-culture of *A. oris* with *S. gordonii* expression of fim-P1 was higher ($p = 0.026$) in the presence of root halves than without (Fig. 7).

Discussion

In this experiment, we showed that invasion of bacteria into dentinal tubules is species-specific, and single-species or dual-species contamination lead to different invasion.

For our experiments, *P. intermedia*, *P. gingivalis*, *F. nucleatum*, *A. oris*, *S. sanguinis*, *S. gordonii*, and *P. micra* were selected, because these species are frequently found in infected root canals [7, 14, 19–21]. Primary infections are predominantly associated with fusobacteria, *P. micra*, oral streptococci, and others [19, 22, 23]. Secondary infections are also dominated by anaerobes [22, 24]; the prevalence of *Enterococcus faecalis* has been reported in some cases [19, 25].

While reference strains were used, differences to clinical isolates and other laboratory strains cannot be excluded. In the case of *S. gordonii* ATCC 10558, we were unable to amplify regions of *sspA* and *sspB* by designing primers based on an available database (accession: U40025.1) and *sspB* (accession: U40026.1), which was based on *S. gordonii* M5. Modifications or missing genes can be suggested as the reason for this lack of amplification.

In our study, samples taken from three different regions (inner, middle, and outer dentine) in the horizontal dimension of the middle third of the root (in vertical dimension) were cultured. A breakage point in the root was created to get

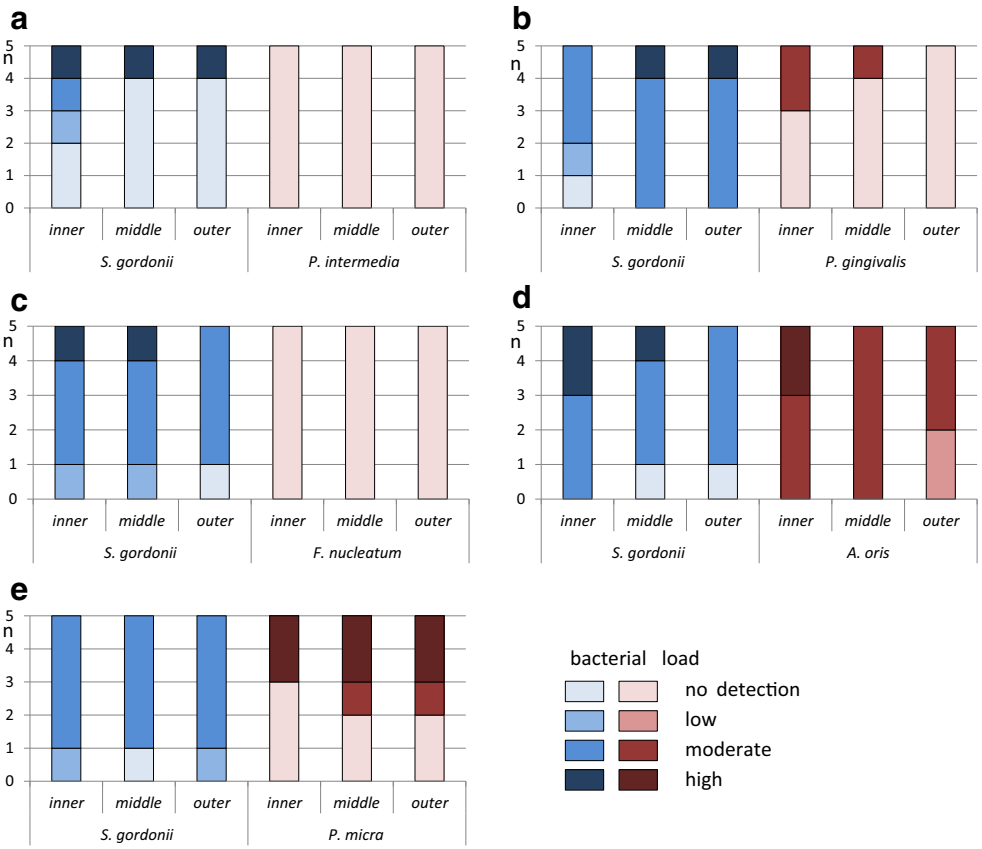

Fig. 3 Categorial data of *Streptococcus gordonii* ATCC 10558 combined with *Prevotella intermedia* ATCC 25611 (**a**), *Porphyromonas gingivalis* ATCC 33277 (**b**), *Fusobacterium nucleatum* ATCC 25586 (**c**), *Actinomyces oris* MG1 (**e**), *Parvimonas micra* ATCC 33220 (**f**) in the inner, middle, and outer part of root dentine after 8 weeks of co-cultivation

samples with the bur and to prepare SEM images. Defects in the breakage of the teeth were shown to not allow accurate observation of the penetration depth [26]. Therefore, we divided the radicular dentine into inner, middle, and outer dentine as described recently [27]. In our study, teeth were incubated continuously for 8 weeks, which should have allowed the bacteria to penetrate deeply. Previous studies have incubated teeth between 10 and 28 days [12, 14, 21, 28].

Our findings for monoculture bacterial strains showed a variation in the depth of bacterial invasion within each strain. As the tooth collection was anonymized, the age of the donors was not available. Depths of invasion are higher at a younger age as diameters of tubules are larger; sclerosis and obliteration may occur in older age [29].

Significant differences between the two streptococcal strains were not observed. *S. gordonii* [18] as well as *S. sanguinis* have been used in laboratory studies [11, 26]. When roots were infected with a single strain, gram-positive strains were found more often in the outer part of the root dentine. This is in accordance with Berkiten et al. [26] who found deep penetration by *S. sanguinis* into dentinal tubules but only limited penetration by *P. intermedia*. In contrast, Perez et al. [14] found no invasion by *P. intermedia* and suggested that the colonies formed by *P. intermedia* and the fibrils

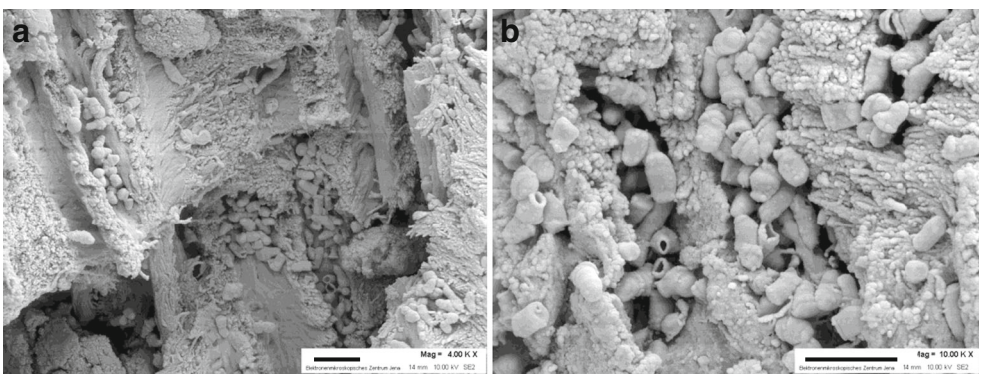

Fig. 4 **a** + **b** SEM of *Actinomyces oris* MG1 in dentinal tubules (bar =2 μm)

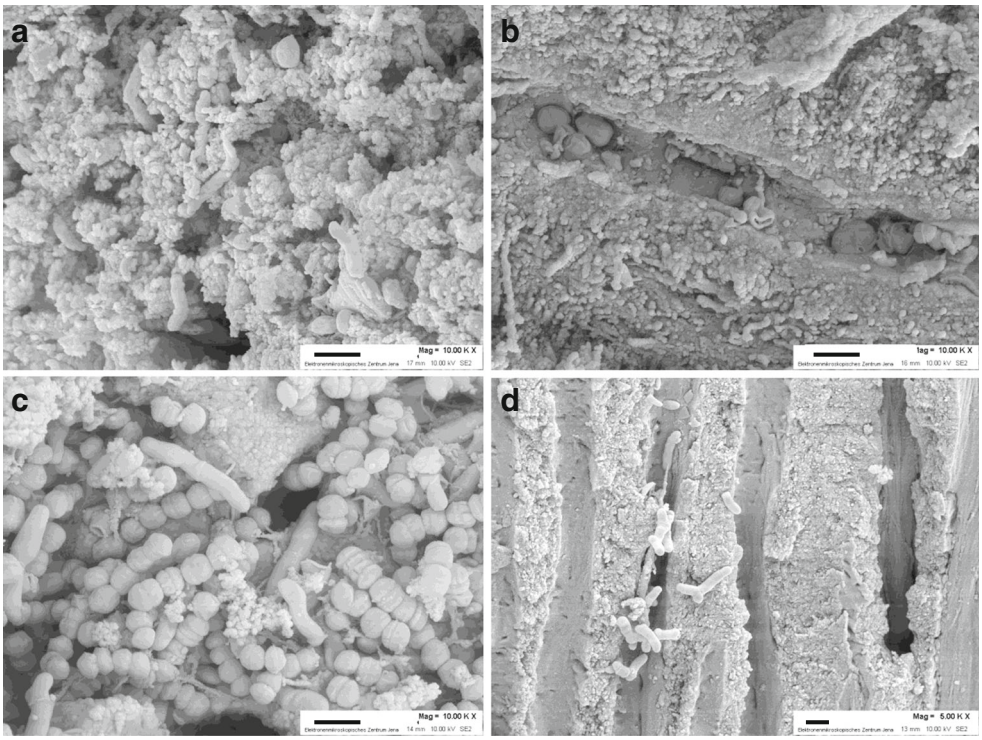

Fig. 5 SEM of two-species infection combining *Streptococcus sanguinis* ATCC 10556 **a**+ **b** with *Fusobacterium nucleatum* ATCC 25586, **c** + **d** with *Actinomyces oris* MG1 around and in dentinal tubules (bar =1 μm)

extending out of the cellular wall of *P. intermedia* prevented penetration.

The invasiveness of *S. gordonii* seems to be much greater when co-cultured with other bacteria, thus confirming the studies of Love et al. [18] who observed that monocultured *P. gingivalis* penetrated dentinal tubules only in combination with *S. gordonii* (not with *S. mutans*) [18]. Differences were explained by the binding of SspA and SspB polypeptides produced by *S. gordonii* to collagen and to *P. gingivalis* [18]. Consistent with these reported results, we found that *P. gingivalis* was able to penetrate dentinal tubules when co-cultured with *S. gordonii* but not in co-culture with

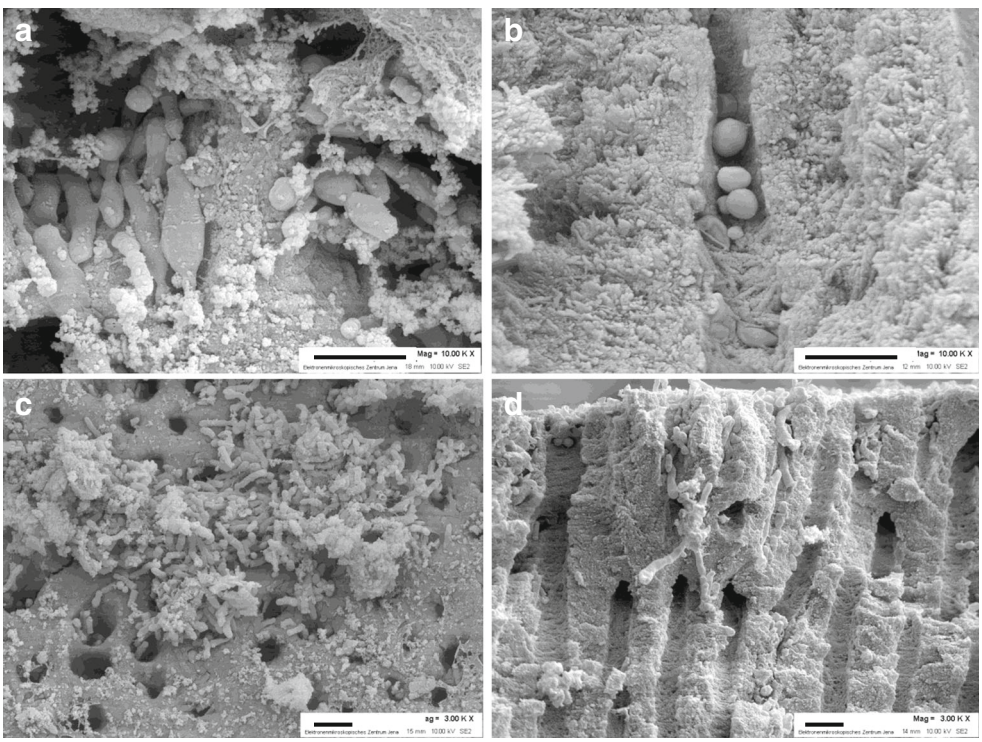

Fig. 6 SEM of two-species infection combining *Streptococcus gordonii* ATCC 10558 **a** with *Fusobacterium nucleatum* ATCC 25586 (the modified swollen shape of *F. nucleatum* is notable), **b** with *Parvimonas micra* ATCC 33220, **c** + **d** with *Actinomyces oris* MG1 around and in dentinal tubules (bar =2 μm)

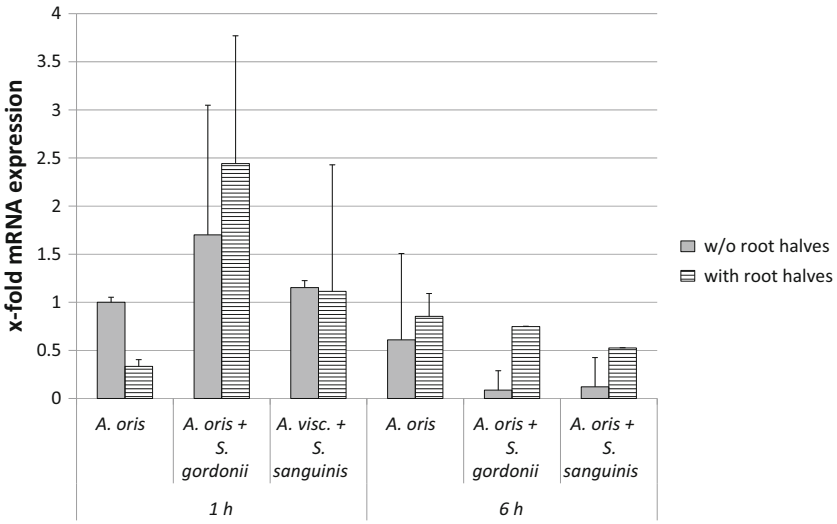

Fig. 7 mRNA expression of *Actinomyces oris* MG1 as a single strain or co-cultured with *Streptococcus sanguinis* ATCC 10556 or *Streptococcus gordonii* ATCC 10558 without or in the presence of root halves (expression is compared to *A. oris*MG1 as a single strain without root halves at 1 h)

S. sanguinis. It should be further investigated which molecules of *S. sanguinis* may inhibit the invasion of co-cultured *P. gingivalis*. When, co-cultured with other bacterial strains, *S. sanguinis* showed, like *S. gordonii*, a greater invasiveness, except when combined with *P. intermedia*.

In contrast to Siqueira et al. [12], we observed no tubule penetration by monocultured *F. nucleatum*. Although growth was not observed in any third of the root dentine, an interesting finding was observed in root canals infected with both *F. nucleatum* and *S. gordonii*. The shape of *F. nucleatum* changed from fusiform to a compressed and plump shape, confirming an observation by Siqueira et al. [12] when exposing it as a single strain to dentine tubules. The high counts of that species observed around the entrances may suggest that the changed shape inhibits the invasion of *F. nucleatum*. The different shape is likely linked to modified gene expression. By contrast, in combination with *S. sanguinis*, *F. nucleatum* did not change its shape and was partially able to invade dentinal tubules. We hypothesized that this resulted from modified expression of *F. nucleatum* adhesin FadA [30], however mRNA expression was not detectable in our experiments (data not shown).

Both in monoculture and in combination with *S. gordonii*, *P. micra* showed penetration to the outer third of the root dentine. But when co-cultured with *S. sanguinis*, no invasion could be observed. In this study, *A. oris* was the most invasive bacterial strain in monoculture and especially in combination with *S. gordonii* and *S. sanguinis*. The bacterial load increased in all thirds of the root dentine in co-culture with streptococci. *A. oris* is a gram-positive, capnophilic bacterium. Gram-positive capnophilic and anaerobic bacterial strains are predominantly found in endodontic treatment failure [19]. The invasion of dentinal tubules by oral bacteria is suggested to be associated with endodontic failure, because these bacteria are protected from endodontic treatment [6, 7]. Furthermore, it is assumed that gram-positive bacterial strains are more resistant to chemo-mechanical endodontic treatment and are better able to adapt to the post-endodontic environment [4].

The generally greater invasiveness of microorganisms when combined with another microorganism suggests a different gene expression pattern. *Actinomyces* sp. synthesizes type 1 fimbriae, which are composed of major FimP subunits (essential in binding to proteins) [31]. Recently it was shown that deletion of *fimP* in *A. oris* MG1 abolished binding to salivary proline-rich proteins [32]. In our study, mRNA expression of FimP in *A. oris* was down-regulated when co-cultured with streptococci after 6 h, however this did not occur in contact with root halves. This indicates that bacteria might react differently with respect to gene expression when in contact with other microorganisms and/or with dentine. In further research, it might be of interest to also study other molecules of *A. oris*, e.g., a polysaccharide containing glucose, mannose, and galactose residues [33], as well as a coaggregation factor A protein [34] which are involved in coaggregation with oral streptococci.

Our results showing the high degree of invasiveness of bacteria, especially when co-cultured, are in agreement with in vivo studies. Using immunohistochemistry in extracted teeth, *F. nucleatum* and *P. micra* were detected in the dentinal tubules of approximately 50 % of the teeth; *P. gingivalis* and *P. intermedia* were found in approximately 30 % of the teeth [7]. Peters et al. [27] also detected mainly gram-positive bacteria in the outer third of dentine by culturing.

The ability to invade dentinal tubules from the cementum should also be taken into account in patients with periodontitis. Bacteria were mostly detected in dentinal tubules up to a depth of 300 μm, sometimes deeper [35]. *P. gingivalis*, *P. micra*, and others were identified both in root canals and in subgingival biofilms [36, 37]. The genotype similarity of anaerobic bacteria in periodontal and endodontic lesions may support the pulp-infection pathway originating from a periodontal infection [37, 38].

This in vitro study has several limitations. No data about the age and the periodontal status of the donors were available. Preparation and storage of the teeth might affect dentine including its binding sites for bacteria. The used bacterial strains were all laboratory strains. In follow-up studies, clinical isolates from endodontic infections should be also included.

However, usually new treatment options for endodontic infections are first evaluated in extracted teeth. Commonly, experiments employ a short incubation time to contaminate roots [39, 40], and in most experiments merely single species are evaluated [39, 40]. Two recommendations can be provided from the present study: firstly, it is strongly suggested to use mixed infection models and secondly, longer incubation times are needed to allow for deep bacterial invasion.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent For this type of study, formal consent is not required.

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